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Interaction of purified alternative oxidase from thermogenic *Arum maculatum* with pyruvate

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ABSTRACT

Plant alternative oxidase (AOX) activity in isolated mitochondria is regulated by carboxylic acids, but reaction and regulatory mechanisms remain unclear. We show that activity of AOX protein purified from thermogenic *Arum maculatum* spadices is sensitive to pyruvate and glyoxylate but not succinate. Rapid, irreversible AOX inactivation occurs in the absence of pyruvate, whether or not duroquinol oxidation has been initiated, and is insensitive to duroquinone. Our data indicate that pyruvate stabilises an active conformation of AOX, increasing the population of active protein in a manner independent of reducing substrate and product, and are thus consistent with an exclusive effect of pyruvate on the enzyme's apparent V_{max}.

1. Introduction

The alternative oxidase (AOX) is a non-proton motive quinol oxidase located in mitochondria of higher plants, pathogenic fungi and some parasites including *Trypanosoma brucei* [1] and *Cryptosporidium parvum* [2,3]. The structure and mechanism of AOX have yet to be fully established, although two independent EPR studies have demonstrated that the AOX active site comprises a binuclear iron centre [4,5]. Recent persuasive FTIR evidence [6] places the AOX firmly in the class of membrane-bound diiron carboxylate proteins [7].

An important insight in AOX function has been obtained from studies with isolated plant mitochondria showing that certain carboxylic acids strongly stimulate AOX activity [8–10]. In mitochondria of non-thermogenic species, such as those isolated from soybean cotyledons [9] and tobacco leaf [11], AOX activity is for example almost fully dependent on the presence of pyruvate. Glyoxylate and other α-keto-acids can substitute for pyruvate [8–10], whilst succinate, instead of pyruvate, is able to activate AOX in tomato [12] and the thermogenic plant sacred lotus [13]. Mitochondria isolated from spadices of thermogenic *Arum maculatum* exhibit both pyruvate-stimulatable and constitutive AOX activities with pyruvate insensitivity increasing as thermogenesis progresses [14].

The molecular interaction between AOX and pyruvate is proposed to occur through formation of a thiohemiacetal with the proximal Cys (Cys I) of two key Cys residues [15,16] that are conserved amongst the sequences of many pyruvate-sensitive AOX enzymes. This thiohemiacetal has been proposed to cause a charge-induced conformational change that is required for stimulated AOX activity [16]. The presence of a Ser at this Cys position results in isozymes that are not stimulated by pyruvate but by succinate instead [12,13,17,18]. Importantly, our recent studies with the *Sauromatum guttatum* AOX (Sg-AOX, i.e., a thermogenic isozyme containing both these Cys residues) have suggested a number of additional conserved sequence elements that are likely to be involved in pyruvate binding [19].

Despite the advances in structural understanding of the AOX–pyruvate interaction, it remains unclear mechanistically how exactly pyruvate stimulates AOX activity. The observation that pyruvate activates AOX only when the mitochondrial ubiquinone pool is highly reduced led to the assertion that pyruvate increases

Abbreviations: AOX, alternative oxidase; deoxyBigCHAP, *N*,*N*-bis(3-gluconamidopropy) deoxycholamide; DTT, dithiothreitol; DQ, duroquinone; DQH_2, reduced duroquinone; EDT-20, *N*,*N*′,*N*′-polyoxyethylene(10)-*N*-tallow-1,3-diaminopropane; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

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the relative affinity of AOX for its reducing substrate, ubiquinol [20]. Since the finding of Zhang et al. [21] that pyruvate was required during purification of the enzyme, it has also been argued, however, that pyruvate has an exclusive effect on maximum AOX activity [22]. In an attempt to reconcile these two observations, Hoefnagel and Wiskich [23] proposed a model whereby pyruvate protected against inactivation of the enzyme by its oxidised quinone product. We considered it important to test this hypothesis experimentally.

In this paper we shed light on AOX regulation using highly active and exceptionally stable AOX protein that we purified from thermogenic *A. maculatum* spadices. We show that duroquinol (DQH_2) oxidising activity of this purified thermogenic AOX is sensitive to pyruvate and glyoxylate but not succinate. This sensitivity is due to an irreversible enzyme inactivation in the absence of pyruvate. Inactivation does not require DQH_2 oxidation and is insensitive to duroquinone (DQ) formation. Our results thus indicate that pyruvate stabilises purified AOX protein independently of reducing substrate and product and, as such, support the notion that α-keto acids have an exclusive effect on the apparent V_{max} of AOX.

2. Materials and methods

2.1. Chemicals

N,*N*-Bis(3-gluconamidopropy) deoxycholamide (deoxyBIGCHAP) was obtained from ICN Pharmaceuticals (Hampshire, UK). All other chemicals were supplied by Sigma (Poole, Dorset, UK).

2.2. AOX purification

AOX was purified from *A. maculatum* mitochondria as described previously [24]. In brief, mitochondria were isolated from thermogenic *Arum* spadices in the presence of 2 mM pyruvate, (see [14]), frozen at −20 °C and stored at −70 °C. On days of purification, it was confirmed that mitochondrial activity had been fully retained during storage. Sub-mitochondrial particles (SMPs) were prepared from these freeze-thawed mitochondria by osmotic shock [24] and subsequent AOX purification was based on [21] with the modifications we described before [24]. AOX was solubilised from SMPs using deoxyBIGCHAP and then purified in a single step by DEAE sepharose-based chromatography. AOX was eluted from the column (GE Healthcare) with 0.5% w/v deoxyBIGCHAP. Note that pyruvate (10 mM) and dithiothreitol (DTT) (5 mM) were present in all purification media. AOX samples were then aliquoted and stored at −80 °C until use. Samples retained activity that was fully sensitive to octyl gallate and insensitive to myxothiazol for at least 1 year.

2.3. Assays

AOX activity was determined either polarographically following oxygen uptake, or spectrophotometrically by simultaneously monitoring changes in reduced and oxidised DQ concentrations All assays were performed in medium D (25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-sodium hydroxide (Tes-NaOH) pH 6.8, 0.025% v/v *N*,*N*′,*N*′-polyoxyethylene(10)-*N*-tallow-1,3-diaminopropane (EDT-20)) in the presence or the absence of organic acids.

DQH_2 was prepared by reducing DQ with sodium dithionite as described in [25]. Solid DQH_2 was stored protected from light at room temperature and was resuspended in acidified (10 mM HCl) ethanol on the day of use. Stock concentrations were determined spectrophotometrically using the following extinction coefficients: 17.8 $mM^{-1}\,cm^{-1}$ at 259 nm for DQ in ethanol [26] and 2.15 $mM^{-1}\,cm^{-1}$ at 283 nm for DQH_2 in water [27]. DTT was not present as standard during DQH_2 oxidation assays to prevent re-reduction of DQ by DTT, however covalent dimerisation state of AOX (assessed by immunoblotting) was unchanged under all assay conditions.

Oxygen consumption was measured at room temperature using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK). Data were recorded digitally using a PowerLab/4SP system (ADInstruments Pty, UK) with Chart version 3.6s software (ADInstruments). AOX (0.3–0.4 μg) was added to 1 ml Medium D. Organic acids (pyruvate, succinate, glyoxylate; all added as sodium salts) were subsequently added from 100-fold stocks dissolved in 25 mM Tes-NaOH, pH 6.8. Reactions were started by addition of 250 μM DQH_2.

For spectrophotometric assays, time-resolved absorbance was monitored at 250 and 290 nm with a Cary 400 Scan UV–Visible spectrophotometer (Varian Analytical Instruments, UK) and experimental progress curves were deconvoluted as described previously to allow initial rate calculations [24]. Extinction coefficients used were 7.10 and 0.534 $mM^{-1}\,cm^{-1}$ for DQ (in medium D) and 0.334 and 1.62 $mM^{-1}\,cm^{-1}$ for DQH_2 (in medium D) at 250 and 290 nm, respectively. All assays were performed using a semi-micro quartz cuvette suitable for magnetic stirring (Hellma UK) thermostated at 20 °C (i.e., room temperature). AOX activity was determined in 700 μl medium D in the presence or the absence of pyruvate as described in the Figure legends. Reactions were started by adding either AOX (0.3–0.4 μg protein, unless otherwise stated) or 250 μM DQH_2.

3. Results and discussion

3.1. Dependency of purified AOX activity on different organic acids

First, we investigated the dependency of purified AOX on pyruvate and other organic acids following AOX-dependent oxygen consumption under different incubation conditions. We and others have shown previously that purified AOX requires a combination of

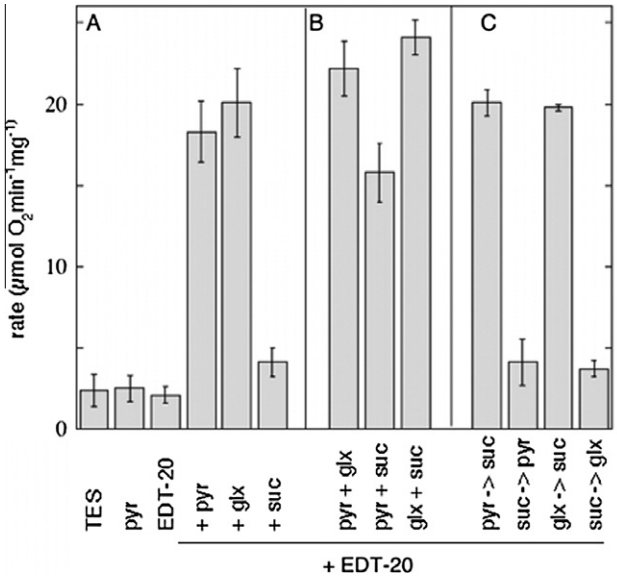

Fig. 1. Dependence of purified AOX oxygen consumption activity on the detergent EDT-20 and on organic acids. Purified AOX (0.3 μg protein) was added to 1 mL of 25 mM Tes-NaOH pH 6.0 in the presence or absence of 0.025% v/v EDT-20. Organic acids were added (10 mM) separately (panel A), in combination (panel B) or sequentially at 30 s intervals (panel C). Reactions were started by addition of 250 μM DQH_2. Pyr, pyruvate; glx, glyoxylate; suc, succinate. Data represent the mean ± S.E.M. of 8–10 replicate polarographic measurements.

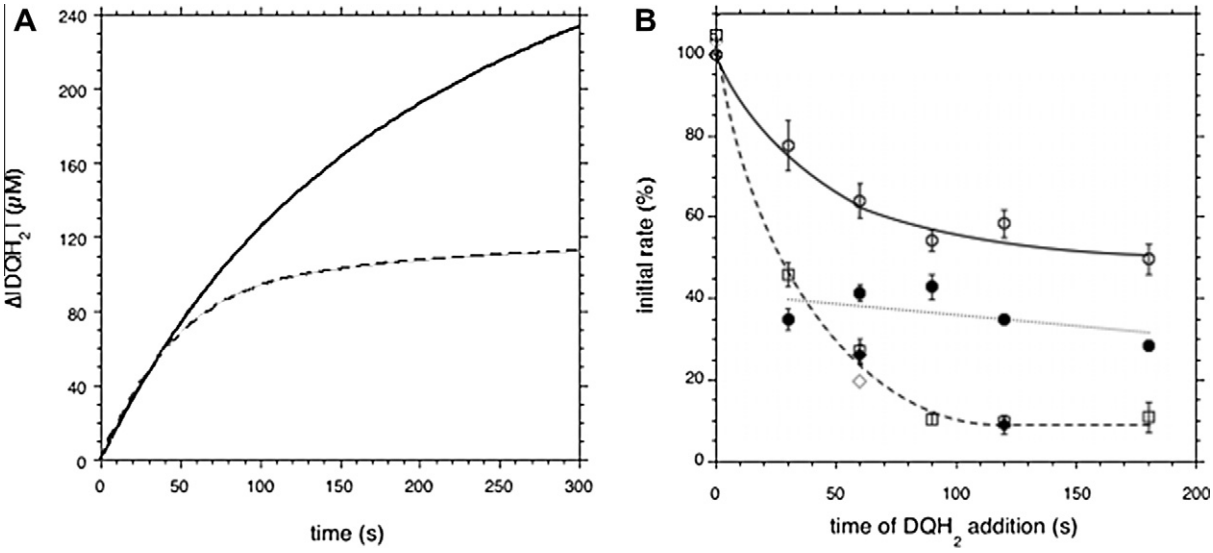

Fig. 2. Inactivation of purified AOX in the absence of pyruvate occurs in the presence (A) and absence (B) of substrate turnover and can be prevented, but not reversed, by the addition of pyruvate. (A) Representative progress curves of duroquinol (DQH_2) oxidation for 0.35 μg of purified AOX in the presence (solid line) and absence (dashed) of pyruvate. Progress curves were derived from the simultaneous spectrophotometric determination of DQH_2 oxidation and DQ reduction [24] in 25 mM TES-NaOH pH 6.0, 0.025% v/v EDT-20, ±10 mM sodium pyruvate and with 250 μM DQH_2 as a substrate. Reactions were started by the addition of AOX protein. Initial rates of DQH_2 oxidation were 201.8 ± 6.8 and 218.6 ± 13.1 μmol DQH_2 min^{-1} mg^{-1} in the presence and absence of pyruvate, respectively ($P = 0.22$, single factor ANOVA). (B) AOX was added at t0 and pre-incubated, without substrate, in the presence (—○—) or absence (- - □ - -) of 10 mM pyruvate. Reactions were started at the times indicated by addition of 250 μM DQH_2 and initial rates of activity calculated. To determine reversibility of inactivation, pyruvate was added 30 s after AOX (- - ● - -). Succinate present from t0 (♦) or added following 30 s pre-incubation (◇) failed to prevent inactivation. Data represent means ± S.E.M. of 2–4 duplicate measurements on 1–2 separate AOX preparations and are standardised to the initial rate of AOX activity in the presence of 10 mM pyruvate at t0 for each enzyme preparation.

detergent (EDT-20) and pyruvate for full activity: lack of either pyruvate or EDT-20 results in low activity [21,24]. The results presented in Fig. 1A confirm this notion demonstrating that pyruvate or glyoxylate (10 mM) increase AOX-mediated oxygen consumption rates some eightfold in the presence of EDT-20. Succinate (10 mM), on the other hand, does not have a significant effect.

Glyoxylate-facilitated AOX activity, both with and without additional pyruvate or succinate, tends to be somewhat higher than activity mediated by pyruvate alone (Fig. 1A and B), suggesting a greater sensitivity of AOX to glyoxylate than pyruvate. Succinate does not affect pyruvate-mediated AOX activity significantly. Of particular interest is the observation that a 30 s pre-incubation with succinate prevents AOX activity mediated by either pyruvate or glyoxylate (Fig. 1C). This finding suggests either that AOX is unstable in the absence of pyruvate or glyoxylate or that succinate prevents interaction of the protein with pyruvate and glyoxylate.

3.2. AOX is irreversibly inactivated in the absence of pyruvate, irrespective of enzyme turnover

To obtain insights into the effects of pyruvate on the quinol-oxidising activity of purified AOX, we next exploited our previously developed spectrophotometric assay to simultaneously measure DQH_2 oxidation and DQ appearance [24]. Fig. 2A shows time-resolved DQH_2 oxidation by AOX in the presence and the absence of pyruvate. When reactions were started by the addition of substrate, initial AOX activities determined spectrophotometrically were similar ± pyruvate (201.8 ± 6.8 and 218.6 ± 13.1 μmol DQH_2 min^{-1} mg^{-1}, respectively). Significant loss of AOX activity is evident, however, during substrate turnover in the absence of pyruvate (Fig. 2A). DTT does not affect this pyruvate effect (data not shown) indicating that AOX inactivation is not due to protein oxidation (*cf.* 'a thiol-regulation' [28]). Together, these observations suggest pyruvate remains bound to AOX both during and following purification and that inactivation occurs when pyruvate is washed out (*cf.* [22]).

We then explored a possible dependence of inactivation on enzyme turnover by incubating AOX ± pyruvate for various times without reducing substrate. The data shown in Fig. 2B reveal that AOX activity decreases significantly following such pre-incubations, both in the presence and the absence of pyruvate. However, whereas a 90 s incubation without pyruvate lowers AOX activity to about 10% of the control rate, the activity in the presence of pyruvate never drops below 60%. Western analysis confirms the reduction status of the AOX dimer is not altered during pre-incubations (data not shown). The findings shown in Fig. 2B thus demonstrate a stabilising role for pyruvate when the enzyme is not turning over. This observation is seemingly at odds with earlier attempts to remove pyruvate from the partially purified AOX by desalting, followed by anaerobic incubation with lactate dehydrogenase, which resulted in only an approximately 40% decrease in AOX activity [23]. However, that finding may have been confounded by maintenance of enzyme-bound quinol in the reduced state due to the presence of NADH-Q oxidoreductase activity in the preparation and incubation with NADH, or differences in the lipid environment of the preparations. Turnover-independent loss of AOX activity is also inferred by the requirement for pyruvate during purification of the enzyme [21,24]. Irrespective of enzyme turnover, the half-time of AOX inactivation is approximately 30 sec (Fig. 2A and B). A similar profile was seen with glyoxylate instead of pyruvate (data not shown) – although rates of DQH_2 oxidation by purified AOX are consistently some 30–40% higher with glyoxylate compared to the same concentration of pyruvate, potentially reflecting different binding affinities and/or subtle differences in the amino acid residues involved in alpha-keto acid binding [16]. While it seems likely that glyoxylate and pyruvate interact similarly with AOX, our data cannot at present exclude the possibility that the mechanistic action of these alpha-keto acids may be subtly different. On the other hand, AOX incubated with 10 mM succinate prior to substrate addition lost activity to the same extent as when pre-incubated in buffer with no added carboxylic acids, suggesting that succinate does not in any way protect the enzyme from inactivation.

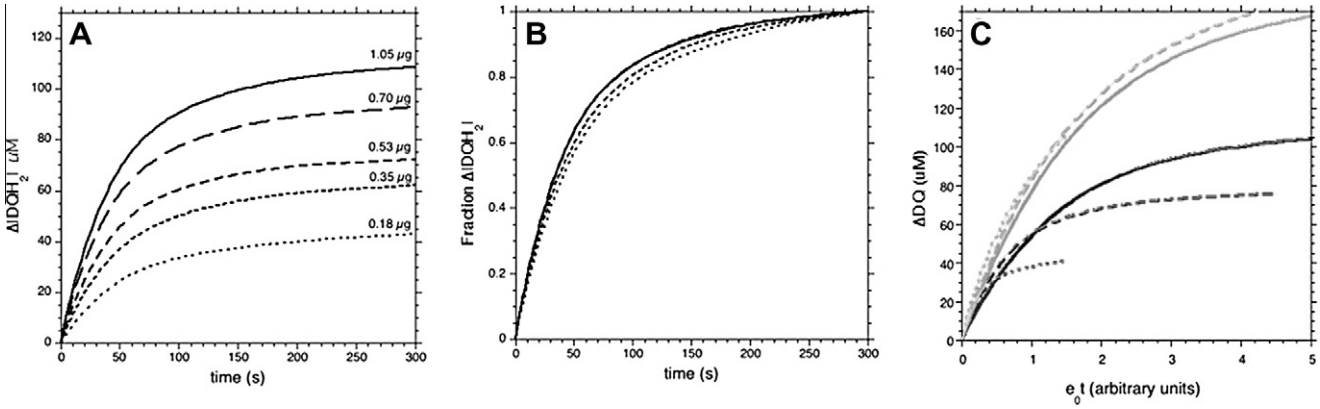

Fig. 3. Pyruvate stabilises an active conformation of AOX in a manner independent of quinone product. (A) Progress curves of DQH_2 oxidation at different amounts of purified AOX. (B) Progress curves standardised to $\Delta|DQH_2|$ at 300 s. (C) Selwyn plot (product concentration as a function of the product of starting enzyme concentration and time) indicates product-independent inactivation of AOX in the absence of pyruvate (black) whilst appreciable inactivation does not occur in the presence of pyruvate (grey). Dots: 0.175 µg; dashes 0.525 µg; solid line 1.05 µg AOX protein. Each curve represents the mean of three duplicate measurements.

It has been established that the stimulating effect of pyruvate on AOX activity is reversible in mitochondria isolated from pyruvate-sensitive species [9]. However, it appears that removal of pyruvate from our purified AOX sample results in an irreversibly inactive enzyme since pyruvate (or glyoxylate – not shown) added 30 s into the pre-incubation period stabilised AOX activity, but failed to recover it (Fig. 2B). Moreover, it is evident that activity of purified AOX is *fully* dependent on pyruvate (or other α-keto acids). This is intriguing given the significant occurrence of both pyruvate-dependent *and* pyruvate-independent AOX activities in thermogenic *Arum* mitochondria and SMPs (data not shown, see also [14]). These discrepancies are perhaps due to lack of phospholipid in the purified sample and may suggest AOX is stabilised in its native environment by the membrane.

3.3. Concentration dependency of AOX for pyruvate

Direct measurement of the dependency of AOX initial rate upon pyruvate concentration was problematic due to the inferred presence of bound pyruvate on the enzyme subsequent to purification, and the irreversible inactivation of the enzyme occurring upon removal of this pyruvate (Fig. 2A and B). The relative affinity of AOX for pyruvate was approximated by pre-incubating the enzyme for 2 min with different pyruvate concentrations and measuring the activity remaining upon subsequent addition of DQH_2 (data not shown). This approach yielded a pyruvate concentration required for half-maximal AOX activity ($K_{1/2}$) of at least 0.5–1 mM, that was unaffected by the presence of succinate. This $K_{1/2}$ is high compared to that of AOX for pyruvate in isolated sub-mitochondrial particles from soybean and skunk cabbage (5–50 µM; [8,10,29]) again probably reflecting differences in the environment of the purified protein. It is noteworthy, however, that the outcome of our experiments was independent of DQH_2 concentration (50–250 µM), suggesting that pyruvate does not affect the interaction of AOX with its reducing substrate and product.

3.4. Pyruvate stabilises an active conformation of purified AOX independent of quinone product

Loss of AOX activity in the absence of pyruvate could be explained in two ways: (1) inactivation secondary to destabilising conformational changes upon pyruvate release and/or (2) product inhibition at lower DQ concentration in the absence than the presence of pyruvate (see [23]). The spectrophotometric data shown in Fig. 3 discriminate between these explanations. Varying the amount of AOX protein in the assay (Fig. 3A) does not affect the time-dependency of enzyme inactivation (Fig. 3B). This observation renders product inhibition an unlikely explanation for loss of AOX activity as one would expect faster build-up of DQ, and hence faster AOX inactivation, when the protein amount is raised. In fact, the half-time of inactivation in the absence of pyruvate (approximately 30 s) is not dependent on enzyme amount. Fig. 3C presents the same protein-dependent data in a way originally suggested by Selwyn [30,31] plotting DQ accumulation against the product of enzyme amount and reaction time. In the presence of pyruvate these 'Selwyn curves' for different AOX amounts overlap (Fig. 3C, grey curves) indicating that activity is proportional to enzyme amount and that there is no appreciable enzyme inactivation during the assay period. The curves are linear to roughly 100 µM DQ at which point AOX activity is inhibited by product. In the absence of pyruvate on the other hand, the 'Selwyn curves' do *not* overlap (Fig. 3C, black curves) and deviate from linearity at different product (DQ) concentrations indicating enzyme inactivation during the assay period. Taken together, the data shown in Fig. 3 support a role for pyruvate in stabilising an active AOX conformation in a manner independent of DQ.

4. Concluding remarks

Various roles for pyruvate in the AOX mechanism have been suggested, including an effect of pyruvate binding on (1) the relative affinity of the enzyme for its reducing substrate [20] and/or product [23], (2) maximum AOX activity (V_{max}) [22] and (3) access of oxygen to the active site [7,16,32]. Interestingly in this respect, we have shown recently that mutation of the conserved distal 'regulatory' cysteine (CysII) to alanine increases AOX's affinity for O_2 [32]. We and others have previously argued that pyruvate interaction with Cys residues could promote conformational changes that primarily affect AOX's affinity for oxygen [7,16,32]). This allosteric interaction may be sufficient to stabilise the purified thermogenic Arum AOX removed from the phospholipid membrane.

We have previously identified novel primary sequence elements that, in addition to the two regulatory Cys residues [15,16], may influence the sensitivity of AOX to pyruvate [19]. Recently, AOX activity in mitochondrial isolated from the thermogenic plant sacred lotus (*Nelumbo nucifera*) was found to be insensitive to pyruvate and glyoxylate, but instead activated by succinate. Analysis of two sacred lotus sequences revealed that – similar to other succinate-sensitive AOX [12] – the regulatory Cys_1 residue was lacking, being Ser instead [13]. Notably,

however, these sacred lotus isoforms contain Q/ENV motif in region 3 (as designated in [19]), one of several sequence regions we have previously hypothesised to be important in sensitivity to pyruvate [19]. Clearly further investigations are required to establish firmly those residues important for specificity of organic acid requirement of AOX in different species.

An important finding from our current data is that the interaction of AOX with pyruvate is independent of quinone product and emphasise the effect of pyruvate on conformational stability. Additionally, our observation that purified AOX activity is irreversibly lost in the absence of pyruvate highlights the importance of the direct membrane/detergent environment for AOX stability. These findings are consistent with an exclusive effect of pyruvate on the V_{max} of AOX (*cf.* [22]). Importantly, however, our data demonstrate that the effect on V_{max} is not due to altered kinetics of the purified enzyme *per se*: pyruvate does not affect the enzyme's catalytic constant (k_{cat}), but instead effectively increases the amount of active protein (e). Our results thus provide important new insights into the still rather elusive AOX mechanism.

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